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Multiple Requirements of the Focal Dermal Hypoplasia Gene Porcupine during Ocular Morphogenesis

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Wnt glycoproteins control key processes during development and disease by activating various downstream pathways. Wnt secretion requires post-translational modification mediated by the *O*-acyltransferase encoded by the *Drosophila* porcupine homolog gene (*PORCN*). In humans, *PORCN* mutations cause focal dermal hypoplasia (FDH, or Goltz syndrome), an X-linked dominant multisystem birth defect that is frequently accompanied by ocular abnormalities such as coloboma, microphthalmia, or even anophthalmia. Although genetic ablation of *Porcn* in mouse has provided insight into the etiology of defects caused by ectomesodermal dysplasia in FDH, the requirement for *Porcn* and the actual Wnt ligands during eye development have been unknown. In this study, *Porcn* hemizygosity occasionally caused ocular defects reminiscent of FDH. Conditional inactivation of *Porcn* in periocular mesenchyme led to defects in mid- and hindbrain and in craniofacial development, but was insufficient to cause ocular abnormalities. However, a combination of conditional *Porcn* depletion in optic vesicle neuroectoderm, lens, and neural crest–derived periocular mesenchyme induced severe eye abnormalities with high penetrance. In particular, we observed coloboma, transdifferentiation of the dorsal and ventral retinal pigment epithelium, defective optic cup periphery, and closure defects of the eyelid, as well as defective corneal morphogenesis. Thus, *Porcn* is required in both extraocular and neuroectodermal tissues to regulate distinct Wnt-dependent processes during morphogenesis of the posterior and anterior segments of the eye. *(Am J Pathol 2015, 185: 197–213; http://dx.doi.org/10.1016/j.ajpath.2014.09.002)*

Focal dermal hypoplasia (FDH) or Goltz syndrome (OMIM #305600) is an X-linked dominant syndrome resulting from defective development and interaction of ectodermal and mesodermal tissues.[1,2] FDH patients exhibit variable manifestations of skin hypoplasia, hypodontia, skeletal abnormalities (including limb and digit defects, as well as reduced bone density), and defects in ocular, kidney, and abdominal wall development. FDH is caused by mutations in the porcupine homolog (*Drosophila*) gene (*PORCN*), which encodes for a highly conserved transmembrane *O*-acyltransferase localized to the endoplasmic reticulum.[3–5] In mouse, PORCN-mediated palmitoylation is critical for trafficking and signaling activity of Wnt proteins, a family of highly conserved cysteine-rich glycoproteins.[6,7] Although Wnt-independent activity of PORCN has been reported in some cases,[8] a systematic analysis revealed that all Wnt proteins require palmitoylation by PORCN for their secretion.[9] Several human developmental disorders have been linked to mutations in Wnt pathway components.[10]

Most FDH patients are heterozygous female with mosaic PORCN function, and the variable phenotypes are possibly due to individual X-chromosome inactivation. Approximately 10% of FDH patients are male, with postzygotic mosaic mutations. Studies in mouse revealed that PORCN is strictly required during gastrulation; thus, zygotic *Porcn* mutations are

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most likely lethal.[11–14] Furthermore, zygotic deletion of the paternal *Porcn* allele in mouse accurately recapitulates the phenotypic mosaicism observed in female patients and results usually in perinatal lethality.[11,15] Although FDH is considered a rare disease, with a prevalence of 1:1,000,000 based on the number of observed live births, studies in mouse suggest that prenatal lethality may affect up to 98% of *PORCN* mutant individuals, implying a prevalence of 1:25,000.[15] Thus, FDH may affect embryonic survival much more significantly than has been acknowledged.

PORCN is expressed in the developing mouse eye and surrounding tissues, and FDH patients frequently exhibit congenital eye defects, including microphthalmia, anophthalmia, colobomata (iris, choroid, retina, and optic nerve), aniridia, and pigment abnormalities.[1,3,12,16] During normal eye development, morphogenesis of the optic cup is a critical step that involves invagination of the distal optic vesicle and overlying surface ectoderm. The resulting inner layer of the optic cup develops into the neural retina, whereas the outer layer gives rise to the retinal pigment epithelium (RPE). The ventral optic cup is connected to the forebrain by the optic stalk, and both the ventral optic cup and the stalk invaginate, resulting in formation of the optic fissure. The peripheral rim of the optic cup differentiates into the ciliary body and iris in the postnatal mouse. The surrounding periocular mesenchyme consists of multiple cell lineages, both neural crest–derived and mesoderm-derived, and its interaction with the adjacent neuroepithelium and lens ectoderm is critical for differentiation of the anterior segment, patterning of the RPE, and optic stalk. Developmental problems during these processes are likely to cause the severe congenital ocular abnormalities observed in FDH.

Although the specific role of *Porcn* during eye development is unknown, interference with downstream components of Wnt pathways in mouse, zebrafish, chick, and frog has revealed that Wnt signaling is critical for diverse processes during eye development. Wnt proteins bind to several surface receptors, including the Frizzled family of transmembrane proteins, and activate several different pathways. In mice and humans, 19 Wnt ligands and 10 Frizzled receptors have been identified. The best characterized is the canonical Wnt–β-catenin pathway, which functions through stabilization of β–catenin, its translocation into the nucleus, and activation of TCF/LEF transcription factors. The role of the Wnt–β-catenin pathway during eye development in vertebrates is often context- and species-dependent, with functions in coordinating retinal progenitor proliferation and differentiation; development of the RPE, lens, ciliary body, and iris; and ocular angiogenesis.[17–38] For example, we and others have shown that Wnt–β-catenin signaling is required for differentiation of the RPE in the mouse optic cup, most likely by direct interaction of TCF/LEF with enhancers of the key regulatory genes *Mitf* and *Otx2*.[20,21,25]

In noncanonical Wnt signaling, activation of Frizzled receptors leads to an increase in intracellular calcium and activation of PKC and CaMKII (Wnt/Ca^{2+} pathway) or activation of small GTPases (RHO, RAC1, CDC42) and JNK, with participation of VANGL and DAAM (PCP pathway).[39] In addition, noncanonical Wnt proteins such as WNT4, WNT5A, and WNT11 can activate receptors other than Frizzled (eg, ROR, RYK).[40] Studies in frog and zebrafish indicate that noncanonical Wnt signaling is essential for formation and/or maintenance of the eye field.[38,41–44] In mouse, disruption of PCP effectors encoded by the *Fuz*, *Wdpcp*, and *Int* genes cause anophthalmia (*Fuz, Wdpcp*) and coloboma (*Int*), respectively; however, the underlying cellular defects are unknown.[45–47]

Several Wnt proteins are robustly expressed in ocular and periocular tissues, such as WNT2B, WNT3, WNT4, WNT5A, WNT5B, WNT7B, and WNT11 in the optic cup, lens, or surface ectoderm and in the periocular mesenchyme.[48,49] Recent observations in chick, frog, and zebrafish demonstrate that WNT2B, WNT3A, WNT4, and WNT11 regulate eye field formation and development of the RPE and the lens.[37,41,42,44,50,51] Interestingly, TGF-β signaling can act cooperatively or adversely with Wnt proteins to regulate different processes of eye development, as recently shown in chick, suggesting that interference with Wnt signaling may also affect other pathways.[37,50,51] To date, however, no ocular defects resulting from deficiency of particular Wnt proteins have been described in mammals.

Redundancy among Wnt ligands, crossregulation between noncanonical and canonical Wnt pathways, and overlap of downstream components with other pathways (and the lack of appropriate tools) have made it difficult to analyze the role of Wnt proteins, particularly those acting via the noncanonical pathway in the developing mouse eye.[52] To gain insight into how ocular development is affected in FDH and to understand the role of *Porcn* during eye development in mammals, we disrupted *Porcn* in ocular and extraocular tissues in mouse. Our results demonstrate that PORCN is expressed in neuroectoderm, lens, and neural crest–derived periocular mesenchyme and that it regulates closure of the optic fissure and eyelid, RPE differentiation, and corneal morphogenesis.

Materials and Methods

Mice

Animal handling and procedures were approved by the University of Utah Institutional Animal Care and Use Committee. The generation of mice carrying the floxed *Porcn* allele [$Porcn^{lox/lox}$; kindly provided by L. Charles Murtaugh (University of Utah, Salt Lake City, UT)] has been described recently.[13] For the purpose of distinction, male mice harboring the floxed *Porcn* allele are referred to as $Porcn^{lox}/Y$. In all crosses, we maintained a mixed genetic background with C57BL/6 and CD-1 mice (Charles River Laboratories International, Hollister, CA). $Porcn^{lox/lox}$ mice were crossed with *Six3-Cre* mice (kindly provided by Yasuhide Furuta, Riken Center for Developmental Biology, Kobe, Japan),[53] *Wnt1-Cre* mice (Jackson Laboratory, Bar Harbor, ME),[54] *Rx3-Cre* mice

[kindly provided by Milan Jamrich (Baylor College of Medicine, Houston, TX)],[55] *ROSA26R*^{LacZ} mice (Jackson Laboratory),[56] and *Axin2*^{lacZ} mice (Jackson Laboratory).[57] Except as otherwise indicated, wild-type (WT) littermates without a *Cre* allele were used as controls.

Counting for timed pregnancies was started at embryonic day 0.5 (E0.5), the day a vaginal plug was detected. Embryos were genotyped by PCR using limb or tail DNA with primer combinations as follows. The *Porcn* forward primer 5′-TGAGTGCTCAAATCCCAACC-3′ and reverse primer 5′-CCAGCATGTGAAAATGTCAAC-3′ generate *Porcn*^{wt} (685 bp) and *Porcn*^{lox} (762 bp) amplicons, and the reverse primer 5′-GTGTCCACCATGTGCATCTC-3′ combines with the same forward primer to produce the *Porcn* delta (*Porcn*$^{\Delta}$) amplicon (485 bp). Primers for *Six3*-(generic-)*Cre* were forward 5′-TCGATGCACGAGTGATGAG-3′ and reverse 5′-TTCGGCTATACGTAACAGGG-3′; for *Six3*-(specific-) *Cre*, forward 5′-CCCTTACGTCCTTCCTCCTC-3′ and reverse 5′-ATGTTTAGCTGGCCCAAATG-3′; for *Wnt1-Cre*, forward 5′-TAAGAGGCCTATAAGAGGCGG-3′ and reverse 5′-ATCAGTCTCCACTGAAGC-3′; for *Rx3-Cre,* forward 5′-GTTGGGAGAATGCTCCGTAA-3′ and reverse 5′-GTATCCCACAATTCCTTGCG-3′; for *SRY,* forward 5′-GCTGGGATGCAGGTGGAAAA-3′ and reverse 5′-CCCTCCGATGAGGCTGATATT-3′; for *ROSAR26*^{LacZ}, forward 5′-GGAGCGGGAGAAATGGATATG-3′, reverse 5′-GCGAAGAGTTTGTCCTCAACC-3′, and reverse 5′-AAAGTCGCTCTGAGTTGTTAT-3′.

Histology, Immunohistochemistry, Quantitative Analysis, and *in Situ* Hybridization

For histology, embryos were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin according to standard procedures. For immunohistochemical analysis, embryo heads were fixed in 4% paraformaldehyde, cryoembedded, and sectioned (usually at 12 μm). If necessary, cryostat sections were treated for antigen retrieval with hot citrate buffer (pH 6) or 1% Triton X-100. The following primary antibodies or markers were used for immunohistochemistry: BRN-3 (dilution 1:50; sc-6026; Santa Cruz Biotechnology, Dallas, TX), caspase-3 (dilution 1:200; 559565; BD Pharmingen, San Jose, CA), CDC42 (dilution 1:150; 2462; Cell Signaling Technology, Danvers, MA), cytokeratin 12 (dilution 1:50; sc-17101; Santa Cruz Biotechnology), F-actin/phalloidin (dilution 1:500; A12379; Life Technologies, Carlsbad, CA), β-galactosidase (β-gal) (dilution 1:5000; Cappel 855976; MP Biomedicals, Aurora, OH), β-gal [dilution 1:750; a generous gift from Nadean Brown (University of California, Davis)], HES1 (dilution 1:1000; a generous gift from Nadean Brown), phospho-histone H3 (dilution 1:1000; Upstate 06-570; EMD Millipore, Billerica, MA), phospho-c-JUN (dilution 1:500; 3270; Cell Signaling Technology), laminin (dilution 1:2000; ab30320; Abcam, Cambridge, MA), LEF1 (dilution 1:100; C12A5; Cell Signaling Technology), MITF (dilution 1:400; X1405M; Exalpha Biologicals, Shirley, MA), OTX2 (dilution 1:1500; AB9566; EMD Millipore), PAX2 (dilution 1:100; Covance PRB-276P; BioLegend, Dedham, MA), PAX6 (dilution 1:300; AB2237; EMD Millipore), PITX2 (dilution 1:1000; PA1020-100; Capra Science, Angelholm, Sweden), β-tubulin class III (dilution 1:2000; Covance PRB-435P; BioLegend), and VSX2 (dilution 1:300; X1180P; Exalpha Biologicals). These were used in combination with the following secondary antibodies: goat anti-rabbit, goat anti-mouse, or goat anti-rat, conjugated with Alexa Fluor 488, 568, or 647 (dilution 1:1000; Life Technologies), donkey anti-goat conjugated with tetramethylrhodamine isothiocyanate (dilution 1:500; 705-025-147; Jackson ImmunoResearch, West Grove, PA), and donkey anti-sheep conjugated with tetramethylrhodamine isothiocyanate (dilution 1:500; 713-165-003; Jackson ImmunoResearch). For quantitative analysis, the number of caspase-3 and p-histone H3−labeled cells in the central and posterior optic cup was counted in alternating sagittal sections for each eye at E11.5, and counts were analyzed using Student's *t*-test. Whole-mount *in situ* hybridization using digoxigenin-labeled Tbx5 and Vax2 riboprobes was performed as described previously.[18] Except as otherwise indicated, at least three embryos were analyzed per genotype, time point, and marker.

Epifluorescence images were captured using an Olympus (Tokyo, Japan) XM10 camera on an upright Olympus BX51 microscope and were processed using Adobe Photoshop CS3 software. Confocal images were captured using an Olympus FV1000 system and were processed using ImageJ version 1.43u (NIH, Bethesda, MD) and Adobe Photoshop CS3 software. All other images were captured using an Olympus MicroFire digital microscope camera U-CMAD3 mounted on the aforementioned BX51 microscope or on an Olympus SZX12 stereomicroscope.

Results

Porcn Hemizygosity Can Result in Diverse Ocular Defects during Embryonic Development

In mouse, male *Porcn*-mutant embryos do not survive beyond gastrulation, because of a failure in mesoderm formation, and female heterozygous mutants typically die perinatally.[11−13] To investigate the role of *Porcn* during ocular development, we performed tissue-specific and temporally controlled inactivation, using females homozygous for a floxed *Porcn* allele and males heterozygous for *Six3-Cre*.[13,53] *Six3-Cre* is activated in the retina and ventral optic stalk at E9.0.[53] Genotyping of extraocular tissues revealed that this cross generated male conditional mutant embryos with two distinct genotypes: *Porcn* mutants harboring a floxed *Porcn* allele that developed normally (*Porcn*^{lox}*/Y*;*Six3-Cre*; $n = 19$) (Figure 1B and Table 1), and, unexpectedly, *Porcn*-mutant embryos with a combination of floxed and delta alleles,

which in rare cases exhibited some developmental abnormalities (*Porcn*$^{\Delta/lox}$*/Y;Six3-Cre in* Table 1). Because *Six3-Cre* can be expressed ectopically,[58] this suggests postzygotic mosaic recombination of the floxed *Porcn* allele.

Most *Porcn*$^{\Delta/lox}$*/Y;Six3-Cre* embryos seemed unaffected (Table 1) and when born showed normal life expectancy. However, when *Porcn*$^{\Delta/lox}$*/Y;Six3-Cre* were used as breeders, they recurrently generated female embryos exhibiting variable ocular and extraocular defects and harboring a combination of recombined and unrecombined *Porcn* alleles (*Porcn*^{het}) (Figures 1 and 2 and Table 1). The observed abnormalities can occur in the absence of *Six3-Cre* (Figures 1C and 2A, and *Porcn*$^{\Delta/lox}$ in Table 1), most likely because of prezygotic deletion of the paternal *Porcn* allele by ectopic CRE activity.[15,58] In accord with previous studies,[11,13,15] we observed extraocular abnormalities with variable frequency and severity, including abnormal craniofacial development and limbs with digit loss and/or fusion (Figure 1C), as well as skin hypoplasia, open ventral body wall, limb atrophy, tail defects (curly or short), and posterior truncation (not shown). Female embryos harboring *Six3-Cre* in addition to recombined and unrecombined *Porcn* alleles exhibited similar defects; however, we could not distinguish between pre- or postzygotic deletion of *Porcn* (Figures 1E and 2C, and *Porcn*$^{\Delta/lox/+}$*;Six3-Cre* in Table 1). Because the developmental defects are consistent across all genotypes, we show here representative examples of female offspring harboring a deleted *Porcn* allele with or without *Six3-Cre*, referred to hereafter as *Porcn*^{het}.

Notably, we detected ocular abnormalities in *Porcn*^{het} embryos that have not been described previously and that may reflect, at least in part, extraocular *Porcn* deletion by ectopic CRE activity. Colobomata were detected in 10% of the embryos ($n = 60$) (Figure 1, C and E and Table 1) and pigment defects of the optic cup with varying severity in up to 42% (Figure 1E and Table 1). To examine the pigment defects in more detail, we investigated whether differentiation of the RPE is disturbed. Normally, RPE specification and differentiation are regulated by the key regulatory transcription factors MITF and OTX2.[59–61] Interference with MITF or OTX2 expression during early eye development results in microphthalmia and coloboma; the RPE transdifferentiates into retina because of a loss of RPE-specific morphology and gene expression, hyperproliferation, and ectopic up-

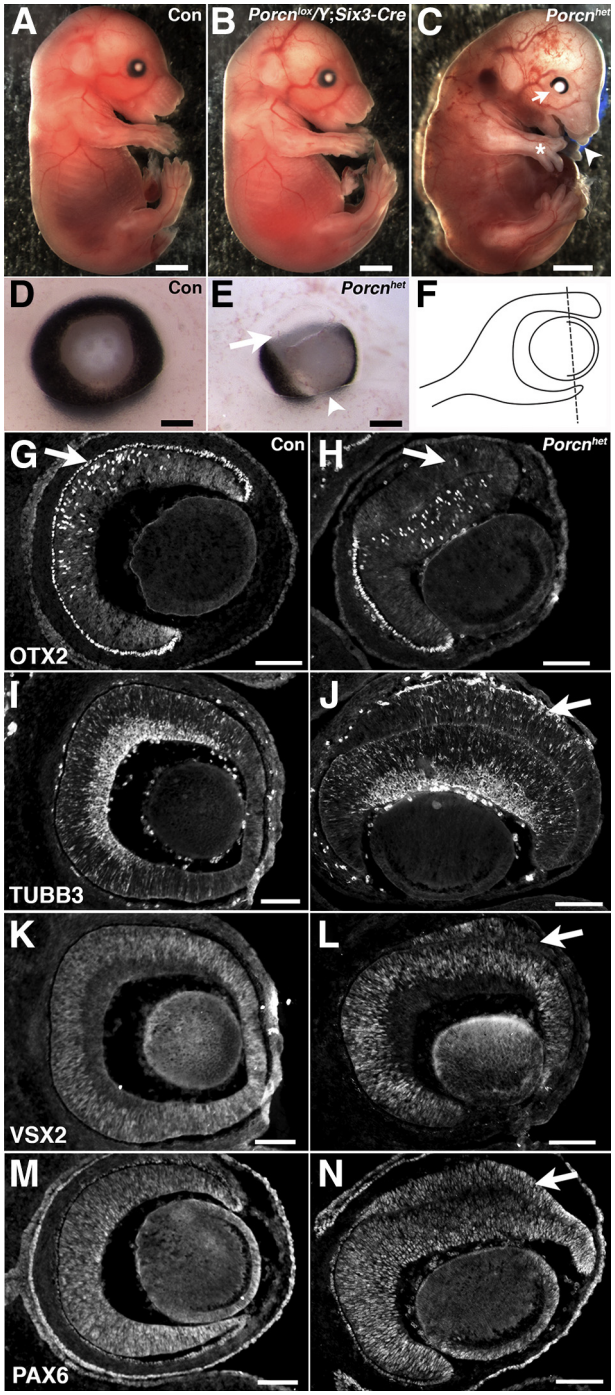


Figure 1 Retinal pigment epithelium (RPE) to retina transdifferentiation and closure defect of the optic fissure in heterozygous *Porcn* female embryos. **A:** Wild-type (WT) embryo at E15.5. **B:** Male embryo with *Six3-Cre*–mediated deletion of *Porcn* (*Porcn*^{lox}*/Y;Six3-Cre*). **C:** Female embryo with heterozygous deletion of *Porcn* in the absence of *Six3-Cre* [genotype: *Porcn*$^{\Delta/lox}$ (*Porcn*^{het})] exhibiting syndactyly and absence of digits in the forelimb (**asterisk**), abnormal craniofacial development (**arrowhead**), and coloboma (**arrow**). **D:** WT eye at E13.5. **E:** *Porcn*^{het} embryonic eye at E13.5 with coloboma (**arrowhead**) and severe pigment loss in the dorsal RPE (**arrow**) (genotype: *Porcn*$^{\Delta/lox}$*;Six3-Cre*). **F:** Location of the sagittal sections in the remaining panels. **G–N:** Immmunohistological analysis of embryos at E13.5. **G:** OTX2 is expressed in the RPE (**arrow**) and, at this age, becomes normally expressed in progenitor cells and photoreceptor precursors in WT retina. **H:** In *Porcn*^{het} embryos, the dorsal RPE has lost widespread expression of OTX2 (**arrow**), but starts to acquire the retina-specific, dispersed pattern of OTX2 expression (genotype: *Porcn*$^{\Delta/lox}$*;Six3-Cre*). **I:** Expression of the retina-specific marker TUBB3 in differentiating neurons in WT retina. **J:** TUBB3 is misexpressed in the dorsal RPE of *Porcn*^{het} eyes (**arrow**) (genotype: *Porcn*$^{\Delta/lox}$). **K:** Expression of VSX2 in retinal progenitor cells in a control eye. **L:** In *Porcn*^{het} eyes, the dorsal RPE is multilayered and VSX2 is ectopically up-regulated (**arrow**) (genotype: *Porcn*$^{\Delta/lox}$*;Six3-Cre*). **M:** In control eyes, PAX6 expression is present in the retina, in the optic cup margins, RPE, anterior epithelium of the lens, and surface ectoderm. **N:** In *Porcn*^{het} eyes, PAX6 is expressed in all ocular tissues, including the dorsal RPE (**arrow**) (genotype: *Porcn*$^{\Delta/lox}$*;Six3-Cre*). Scale bars: 1 mm (**A–C**); 100 μm (**D**, **E**, **G–N**). Con, wild-type control.

Table 1 Ocular and Extraocular Abnormalities in *Porcn*-Mutant Mice at Embryonic Age E12.5 to E18.0

Genotype	OFCD, %	RPE defects,* %			Open eyelid,† %	Cranio-facial defects, %	MHB defects, %	Normal, %	Total embryos, No.
		Severe	Moderate	Mild					
Porcn^{lox}/Y; *Six3-Cre*	0	0	0	0	0	0	0	100	19
Porcn$^{\Delta/lox}$/Y; *Six3-Cre*	0	0	0	7	13	3	3	90	29
Porcn^{het}‡	10	5	18	42	43	28	3	42	60
Porcn$^{\Delta/lox}$	8	4	31	22	20	35	4	31	26
Porcn$^{\Delta/lox/+}$; *Six3-Cre*	12	6	9	53	56	24	3	50	34
Porcn^{lox}/Y; *Wnt1-Cre*	2	0	0	0	0	100	100	0	61
Porcn^{lox}/Y; *Rx3-Cre*	13	0	20	50	0	95	17	0	30
Porcn^{lox}/Y; *Wnt1-Cre*; *Rx3-Cre*	72	52	69	18	100	100	100	0	29
Porcn$^{lox/+}$; *Wnt1-Cre*; *Rx3-Cre*	9	0	9	82	0	0	0	22	23
WT	0	0	0	0	0	0	0	100	109

*Severe RPE defects: embryos with transdifferentiated RPE or almost complete loss of pigment (eg, Figure 1E). Moderate RPE defects: eyes with severe pigment gaps or reduction of pigment circumferentially (eg, Figure 2D). Mild RPE defects: eyes with small gaps or a subtle reduction of pigment circumferentially (eg, Figure 2A), analyzed between E15.5 and E18.0.

†The eyelid closure defect was determined at E16.5 to E18.0.

‡*Porcn*$^{\Delta/lox}$ and *Porcn*$^{\Delta/lox}$;*Six3-Cre* combined.

MHB, Mid- and hindbrain; NE, normal; OFCD, optic fissure closure defect; WT, wild-type.

regulation of retina-specific genes (reviewed by Fuhrmann et al[62]). In the developing eye, OTX2 is normally expressed in both the RPE and retina; however, the expression pattern differs, reflecting different requirements in each tissue.[61,63] In *Porcn*^{het} embryos exhibiting severe pigment defects in the dorsal optic cup, RPE-specific expression of OTX2 is down-regulated (Figure 1, G and H). The affected RPE acquires a retinal fate by up-regulation of the retina-specific genes *Tubb3* and *Vsx2* (Figure 1, I–L), whereas *Pax6* is robustly expressed (Figure 1, M and N). These results indicate that large regions of the RPE in *Porcn*^{het} eyes can trans-differentiate into neural retina.

Porcn^{het} embryos also exhibited a range of less severe pigment abnormalities in the optic cup periphery between E13.5 and E15.5, particularly as unpigmented patches or circumferential pigmentation loss (Figure 2 and Table 1). Unpigmented patches can arise from missing tissue, as shown by interrupted laminin expression, separating two distinct areas of retinal tissue adjacent to the gap (Figure 2, A–C). These gaps may be caused by asymmetrical growth and defective fusion of the optic cup margins. Circumferential pigment loss ranges in severity, resulting from pigmentation defects of the dorsal peripheral RPE (Figure 2D) and absence of tissue in the ventral optic cup periphery (see *Anterior Segment Abnormalities*). Loss of dorsal pigmentation is accompanied by a decrease in expression of the key regulatory genes *Mitf* and *Otx2* (Figure 2, E–H). Interestingly, the retina-specific gene *Tubb3* is not ectopically up-regulated (Figure 2, I and J), in contrast to *Vsx2* (Figure 2, K and L). The HMG box transcription factor *Lef1* can be a target and readout for active canonical Wnt signaling in many tissues, including the developing eye. Normally, LEF1 is expressed in very few cells in the embryonic retina but is robustly present in the peripheral RPE, corneal mesenchyme, and optic cup periphery (Figure 2M).[64] In *Porcn*^{het} embryos, LEF1 is strongly expressed in the dorsal, abnormal RPE periphery suggesting that Wnt–β-catenin signaling is still active (Figure 2N). These observations suggest that the moderately affected RPE periphery does not undergo a complete transdifferentiation into retina, but rather acquires an intermediate fate. The overall morphology of *Porcn*^{het} eyes exhibits other abnormalities; the vitreous is reduced (Figure 2, H and L), and the developing cornea appears thinner (Figure 2, H, L, and N). Furthermore, the ventral optic cup in *Porcn*^{het} embryos can exhibit hypoplasia in the periphery (Figure 2, H, J, L, and N), evident as loss of LEF1 expression (Figure 2N).

Porcn Deletion in Multiple Tissues Is Required to Cause Ocular Defects

So far, our results showed that hemizygosity of *Porcn* can result in variable developmental ocular abnormalities with low penetrance. We hypothesized that a further decrease in PORCN expression would increase the incidence and consistency of ocular defects. In addition, several Wnt proteins are expressed in ocular tissues (optic cup, lens) and surrounding periocular mesenchyme, but it is unclear in which tissue Wnt expression is required.[48,49] Because the periocular mesenchyme provides important factors for optic fissure closure and RPE differentiation,[65–67] we inactivated *Porcn* in neural crest–derived periocular mesenchyme using *Wnt1-Cre* mice (*Porcn*^{lox}/Y;*Wnt1-Cre*[54]) (Supplemental Figure S1A). *Porcn*^{lox}/Y;*Wnt1-Cre* embryos showed no ocular abnormalities (n = 61); however, we observed severe, fully penetrant defects in the mid- and hindbrain region (Figure 3, A and B, and Table 1). This is consistent with deletion of other Wnt pathway components, including WLS (alias GPR177), Wnt1 and Wnt3, Wnt5a, and β-catenin.[68–72] In particular, the integral membrane protein GPR177 could be considered the closest functional Wnt pathway component to PORCN; it is required for proper trafficking and secretion of Wnt proteins and requires PORCN-mediated palmitoylation of Wnt proteins

for recognition.[9,73,74] Mutant embryos with *Wnt1-Cre*–mediated disruption of *Wls* (alias *Gpr177)* fail to form the isthmic organizer, resulting in abnormal mid- and hindbrain development.[68] We therefore hypothesize that the isthmic organizer may not be established properly in *Porcn*^{lox}*/Y*;*Wnt1-Cre* embryos; further studies are needed for confirmation. In addition, *Porcn*^{lox}*/Y*;*Wnt1-Cre* embryos show abnormal development of facial primordia, cleft palate, and a mild, fully penetrant median cleft lip (Figures 3B, 4B, and 4G).[13] Previous studies have demonstrated that inappropriate levels of Wnt–β-catenin and noncanonical Wnt signaling are associated with cleft lip and palate in both humans and mice. Specifically, Wnt proteins are expressed in the mesenchyme of the facial prominences, in the oral ectoderm, and in the palatal shelf mesenchyme and regulate proliferation, cell death, differentiation, and cell migration[75] (reviewed by He and Chen[76]).

Porcn^{lox}*/Y*;*Six3-Cre* embryos did not exhibit any obvious ocular abnormalities (Figure 1B), and it is possible that the timing of *Porcn* inactivation in ocular tissues may be critical. To examine the role of *Porcn* in ocular tissues earlier, we used another eye-specific Cre line, *Rx3-Cre*, that becomes active in the presumptive retina and RPE of the optic vesicle at E8.75 and can be ectopically expressed in the lens vesicle (Supplemental Figure S1B).[55] *Rx3-Cre* is also expressed in the epithelium of the medial nasal prominence.[55] In the present study, *Porcn*^{lox}*/Y*;*Rx3-Cre* mutants (Figure 3C) occasionally exhibited ocular phenotypes such as mild circumferential loss of pigment and coloboma (Table 1). The penetrance was similar to that of *Porcn*^{het} embryos, suggesting that extraocular PORCN expression may be sufficient to prevent a higher incidence of ocular defects. In addition, most of the mutant embryos developed bilateral fusion defects in the upper lip and cleft palate, possibly because of Cre activity in the oral ectoderm (Figures 3C, 4C, and 4H and Table 1).

So far, our observations suggested that PORCN may be required in multiple ocular and extraocular tissues. We therefore performed *Porcn* inactivation using a combination of *Wnt1-Cre* and *Rx3-Cre* lines (Supplemental Figure S1C). Indeed, *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* embryos showed a much higher penetrance of ocular defects and, similar to *Porcn*^{lox}*/Y*;*Wnt1-Cre* embryos, had full penetrance of abnormal mid- and hindbrain development, as well as cleft lip and palate (Figures 3D, 4D, 4I, and 5B and Table 1). In rare, extreme cases, the eyes were almost completely devoid of pigmentation (Figure 3H). Female embryos heterozygous

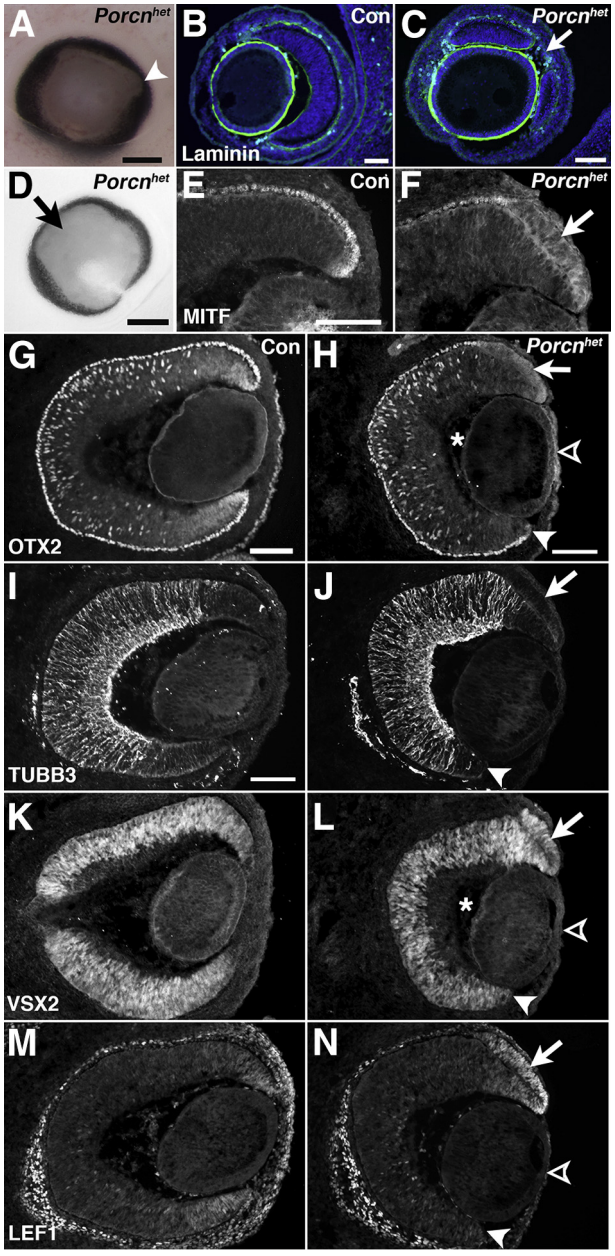


Figure 2 *Porcn*^{het} embryos exhibit pigment defects in the optic cup periphery at E13.5. **A:** *Porcn*^{het} eyes exhibit patches of depigmentation, often found temporally (**arrowhead**) (genotype: *Porcn*$^{\Delta/lox}$). **B:** Sagittal view of the ocular periphery shows continuous, basal laminin expression surrounding the lens and the retina and RPE in WT embryo. **C:** In *Porcn*^{het} embryos, unpigmented patches arise from tissue gaps evident by interrupted laminin expression (**arrow**) (same eye as in **A**). **D:** Colobomatous *Porcn*^{het} eye with circumferential decrease in pigment (**arrow**) (genotype: *Porcn*$^{\Delta/lox}$;*Six3-Cre*). **E:** Coronal view of expression of the RPE-specific protein MITF in WT dorsal RPE. **F:** In *Porcn*^{het} embryos, MITF expression is decreased in the RPE of the optic cup periphery (**arrow**) (same eye as in **D**). **G:** Coronal view of OTX2 expression in WT eye. **H:** OTX2 expression is slightly decreased in the dorsal RPE of *Porcn*^{het} embryos (**arrow**; same eye as in **D**). **I:** Expression of the retina-specific marker TUBB3 in WT retina. **J:** In *Porcn*^{het} eyes, TUBB3 is normally expressed in the retina and absent in the dorsal RPE periphery (**arrow**) (same eye as in **D**). **K:** Coronal expression of the retina-specific transcription factor VSX2 in WT retina. **L:** VSX2 is ectopically up-regulated in the dorsal RPE of *Porcn*^{het} embryos (**arrow**) (same eye as in **D**). **M:** In WT embryos at E13.5, LEF1 is expressed in the periphery of the retina and RPE, and in periocular mesenchyme. **N:** In *Porcn*^{het} embryos, LEF1 expression in the ventral optic cup (**arrowhead**) and in corneal mesenchyme (**open arrowhead**) is absent but robustly up-regulated in the dorsal RPE (**arrow**; same eye as in **D**). **H, J, L,** and **N:** Note the shortening of the ventral optic cup (**arrowhead**), reduction of the vitreous (**asterisks**), and thinning of the cornea (**open arrowhead**) in *Porcn*^{het} embryonic eyes. Scale bars: 200 μm (**A** and **D**); 100 μm (**B**, **C**, **E**, **G**, **H**, and **I**).

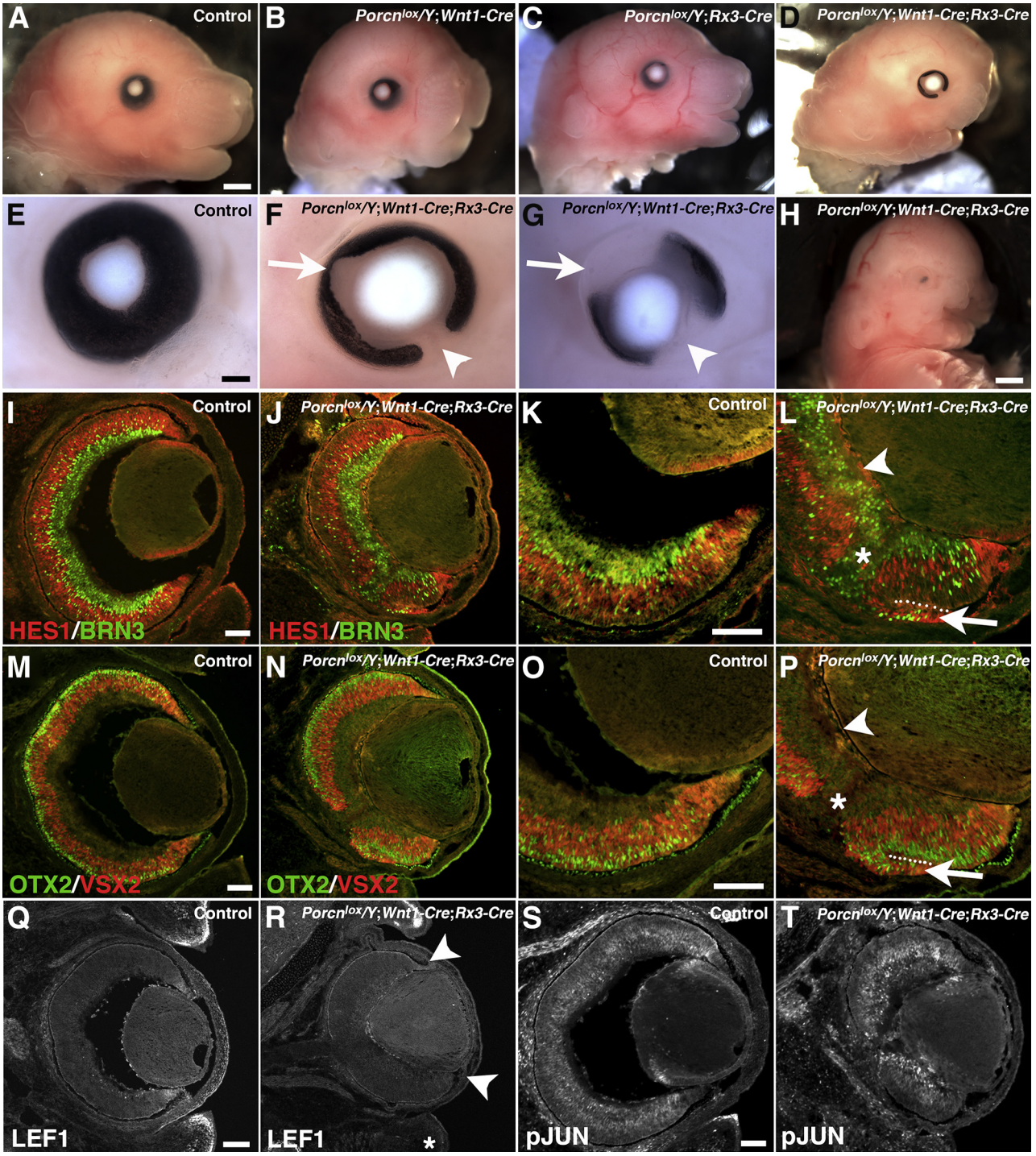


Figure 3 Conditional *Porcn* inactivation in ocular and neural crest–derived mesenchyme induces pigment abnormalities and transdifferentiation of the retinal pigment epithelium (RPE) at E15.5. **A**–**D:** Lateral views of WT (**A**), *Porcn*^{lox}*/Y;Wnt1-Cre* (**B**), *Porcn*^{lox}*/Y;Rx3-Cre* (**C**), and *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos (**D**). **E:** WT eye. **F**–**H:** Range of severities of ocular phenotypes found in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos: colobomata (**arrowhead**) with largely circumferential pigment loss (**F**, **arrow**); more severe pigment loss dorsally (**G**); or, in rare cases, completely absent pigment (**H**). **I**–**T:** Coronal views. **I** and **K:** Expression of HES1 (red) in retinal progenitor cells and BRN-3 (green) in differentiating ganglion cells in WT retina, with WT ventral optic cup shown at higher magnification (**K**). **J:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* retina, expression of HES1 and BRN-3 shows a normal distribution. **L:** Higher magnification of the *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* ventral optic cup reveals ectopic up-regulation of HES1 and BRN-3 in the RPE (**arrow**). The **dotted line** marks the apical borders of retina and transdifferentiated RPE; note optic stalk (**asterisk**) and reduced vitreous (**arrowhead**). **M** and **O:** Expression of OTX2 (green) in retinal progenitors and photoreceptor precursors and of VSX2 (red) in retinal progenitors in WT retina, with ventral optic cup shown at higher magnification (**O**). **N:** Expression of OTX2 and VSX2 appears normal in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* retina. **P:** Higher magnification of the *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* ventral optic cup reveals ectopic up-regulation of VSX2 and retinal OTX2 expression in the RPE (**arrow**); the **dotted line** marks the apical borders of retina and transdifferentiated RPE; note optic stalk (**asterisk**) and reduced vitreous (**arrowhead**). **Q:** LEF1 expression in WT retina. **R:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos, LEF1 is down-regulated in the dorsal and ventral optic cup periphery (**arrowheads**) and in the ventral eyelid mesenchyme (**asterisk**). **S:** p-JUN expression in WT retina and lens. **T:** p-JUN in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos appears normal. Scale bars: 1 mm (**A** and **H**); 200 μm (**E**); 100 μm (**I**, **K**, **M**, **O**, **Q**, and **S**).

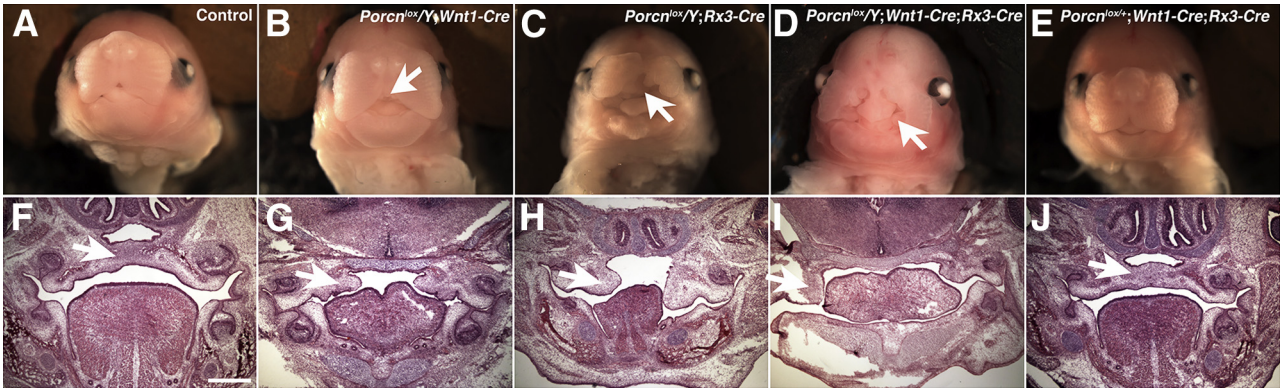


Figure 4 Craniofacial defects in conditional *Porcn*-mutant embryos. Gross (**A**–**E**) and histological (**F**–**J**) images of control and mutant embryos at E15.5. Hematoxylin and eosin stain. **A:** Frontal view of WT embryo. **B:** *Porcn*^{lox}*/Y;Wnt1-Cre* embryos have a mild cleft in the medial region of the upper lip (**arrow**). **C:** *Porcn*^{lox}*/Y;Rx3-Cre* embryos have cleft lips (**arrow**). **D:** *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos exhibit craniofacial abnormalities including severe cleft lips (**arrow**). **E:** Normal craniofacial development in *Porcn*$^{lox/+}$*;Wnt1-Cre;Rx3-Cre* embryos. **F:** In control embryos, a uniform palate is formed (**arrow**). **G:** In *Porcn*^{lox}*/Y;Wnt1-Cre* embryos, the palatal shelves rise horizontally but do not fuse (**arrow**). **H:** *Porcn*^{lox}*/Y;Rx3-Cre* embryos have cleft palate (**arrow**). **I:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos, the palatal shelves do not rise above the tongue and they fail to fuse (**arrow**). **J:** Palatogenesis is normal in *Porcn*$^{lox/+}$*;Wnt1-Cre;Rx3-Cre* embryos (**arrow**). Scale bar = 500 μm.

for conditional *Porcn* inactivation using both Cre lines (*Porcn*$^{lox/+}$*;Wnt1-Cre;Rx3-Cre*) consistently and more frequently (82%) exhibited mild RPE defects (Table 1). Overall, our results demonstrate that the incidence and severity of pigment defects are markedly increased when the level of PORCN expression in retina, RPE, lens, and neural crest–derived mesenchyme is further reduced. (Figure 3, F–H, and Table 1). In addition, affected eyes can appear slightly microphthalmic (Figure 3E). Analysis of the eye perimeter revealed that mutant eyes are 12% smaller than control eyes: 87.84 ± 5.74% in mutants ($n = 18$ eyes from 9 embryos) versus 100 ± 7.98% in controls ($n = 30$ eyes from 16 embryos) ($P < 0.0001$). Further analysis of *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos revealed transdifferentiation of the dorsal RPE into retina, acquisition of intermediate fate or local loss of tissue ($n = 6$ embryos at E15.5) (not shown), which is similar to *Porcn*^{het} eyes and results in a failure to form the ciliary body and iris epithelium (iris hypoplasia; see *Anterior Segment Abnormalities*).

Recently, it has been shown that *Fz5*, *Fz8*, and *Sfrp1/2* compound mutants exhibit accelerated generation of early-born ganglion cells and misregulation of HES1 expression, which is required for progenitor expansion during neurogenesis in the embryonic mouse retina.[77,78] We therefore performed immunohistochemistry for markers labeling different populations of retinal progenitors (HES1, VSX2, OTX2) and early differentiating retinal cell types (BRN-3 for ganglion cells and OTX2 for photoreceptor precursors) in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes. We observed no obvious difference in the developing central retina at E15.5 (Figure 3, J and N, and Supplemental Figure S2), which suggests that retinal neurogenesis at this stage proceeds largely normally, consistent with typical retinal expression of OTX2 and TUBB3 in *Porcn*^{het} eyes (Figure 2, H and J). However, the ventral RPE ectopically up-regulated the retina-specific expression of HES1, VSX2, OTX2, and BRN-3, indicating that it undergoes transdifferentiation into retina (Figure 3, K, L, O, and P). Furthermore, similar to *Porcn*^{het} eyes, the vitreous is strongly reduced in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes, and lens and retina are tightly attached to each other (Figure 3, L and P). Consistent with an effect of *Porcn* disruption on Wnt–β-catenin signaling, we observed a down-regulation of LEF1 in the optic cup periphery and ventral eyelid mesenchyme (Figure 3, Q and R). p-JUN, the putative downstream effector of several signaling pathways (including noncanonical Wnt), appears to be unaffected in ocular and extraocular tissues in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos (Figure 3, S and T).

The Defect in Closure of the Optic Fissure Is Associated with Reduced Wnt–β-Catenin Activity in the Underlying Periocular Mesenchyme

Porcn^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos showed a high incidence of colobomata (72%) (Figure 3, F and G, and Table 1). During normal optic cup morphogenesis, the laterally growing edges of the RPE and retina at the margin of the optic fissure align against each other to fuse and form a continuous optic cup.[79,80] The optic fissure forms as a ventral groove by asymmetric invagination extending from the vitreal side to the proximal junction with the forebrain, which allows mesenchymal cells to migrate inward and form the hyaloid vasculature. Closure of the optic fissure in mouse starts at approximately E10.5 and is complete by E12.5. The etiology of coloboma is complex. Coloboma can result from genetic, extracellular signaling, or environmental perturbations within the optic cup neuroepithelium and periocular mesenchyme. Many genes critical for closure of the optic fissure have been identified in humans and in animal models, including *Pax2*, *Pax6*, *Vax2*, *Pitx2*, *JNK1/2*, and multiple Wnt pathway components[21,77,81,82] (reviewed

by Gregory-Evans et al[83] and Chang et al[84]); however, the cellular mechanisms of the closure defects remain largely unresolved. To gain more insight into the possible mechanisms causing coloboma in *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* embryos, we examined eyes at E12.5, right after completion of optic fissure closure and when any closure defect first becomes obvious. Besides ocular defects, abnormalities in hindbrain and craniofacial development are detectable at this stage (Figure 5, A and B). The most common type of coloboma in *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* mutants was characterized by a narrow gap in the ventral optic cup, suggesting that the defect occurs late during the closure process [9/11 (82%) embryos] (Figure 5, B–D). The closure defect in *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* mutants is confirmed by persistent expression of the basement membrane component laminin (Figure 5, E and F).

Previous studies (eg, Westenskow et al,[21] Martinez-Morales et al,[61] Lee et al,[85] and Tang et al[86]) have shown that genetic models of RPE transdifferentiation can develop colobomata. In *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* embryos at E15.5, the ventral RPE transdifferentiates (Figure 3, L and P), suggesting that improper RPE differentiation could cause the closure defect in the optic fissure. However, we observed that RPE differentiation appears to be normal at E12.5; MITF is present, and VSX2 expression is confined to the ventral retina in *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* embryos (Figure 5, G–J). Furthermore, PAX2 and Vax2 are normally expressed in the ventral optic cup of *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* eyes (Figure 5, I–L). Expression of the dorsal patterning marker Tbx5 is slightly reduced (Figure 5, M and N), which we also observed in *Porcn^{lox}/Y;Rx3-Cre* mutants (not shown) that exhibit a low penetrance of colobomata (Table 1). Apoptotic

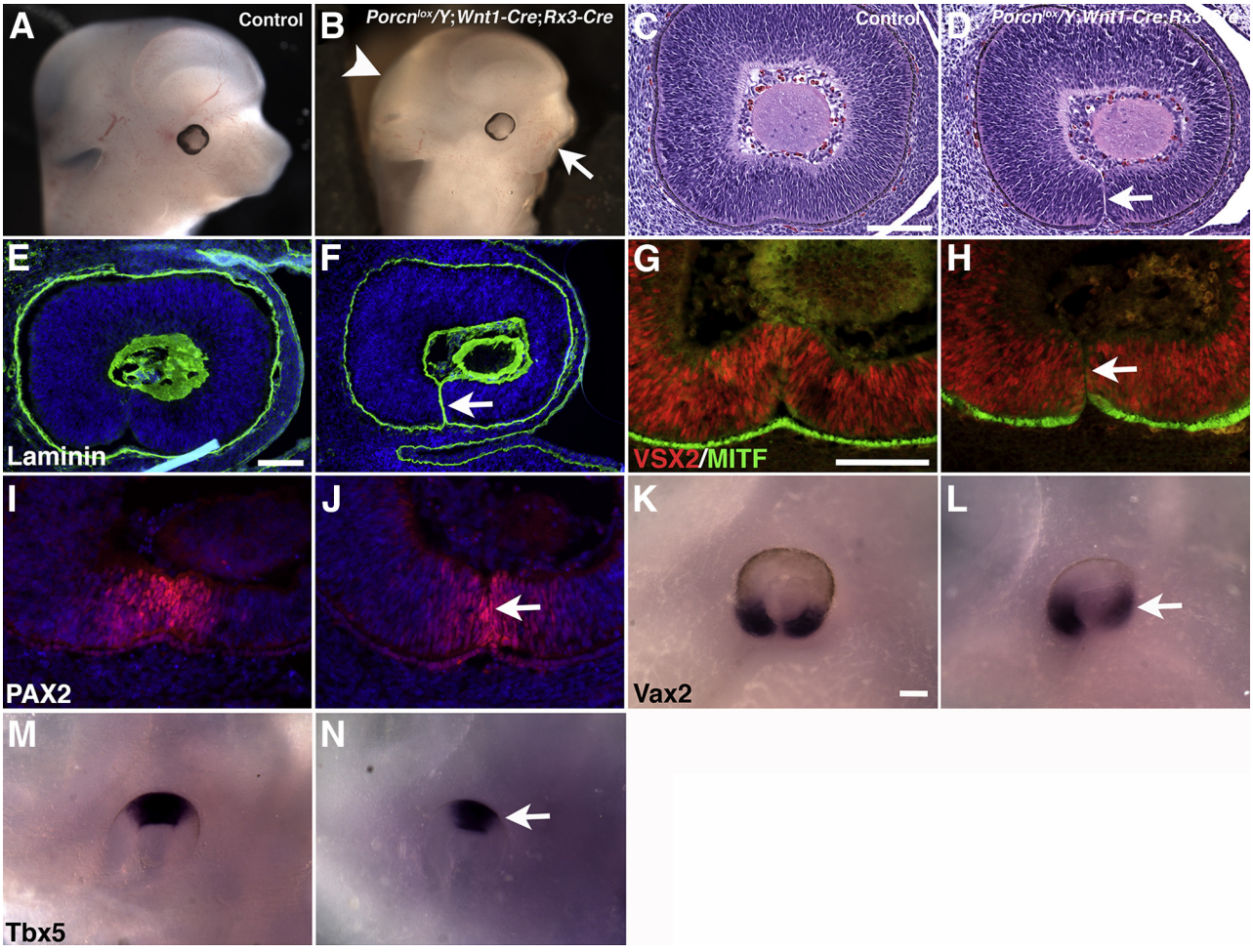
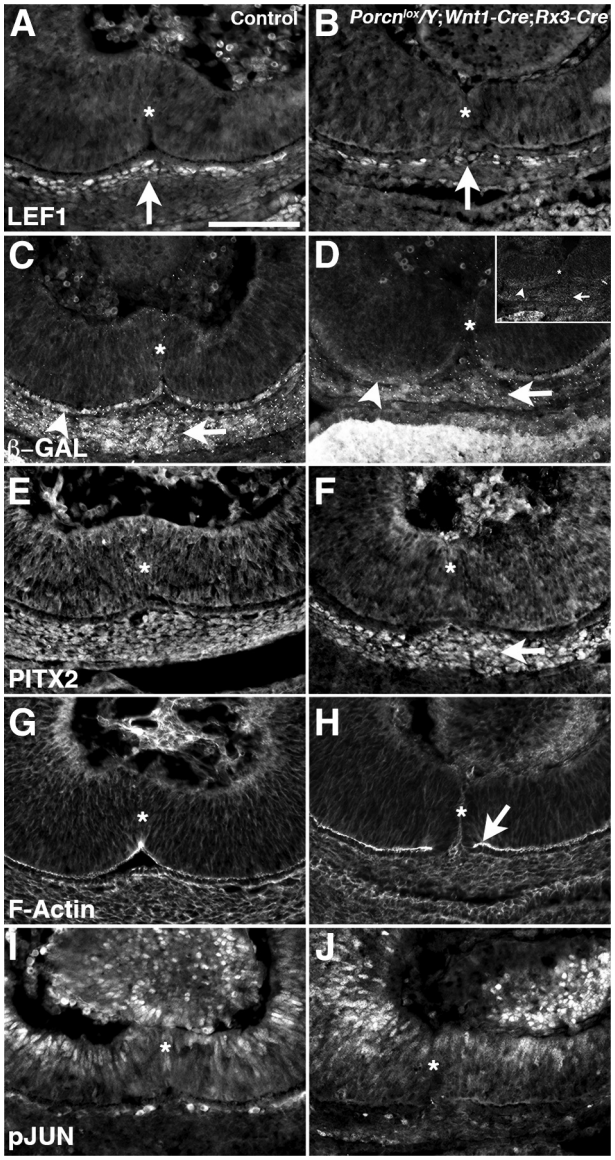


Figure 5 Patterning of the ventral optic cup in *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* eyes. **A**–**J:** Lateral (**A** and **B**) and sagittal (**C**–**J**) views at E12.5. **A:** Control embryo. **B:** *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* embryo shows coloboma as a narrow gap in the ventral optic cup. In addition, defects in craniofacial (**arrow**) and mid- and hindbrain development (**arrowhead**) are detectable. **C:** Control eye. **D:** *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* eye with open optic fissure (**arrow**). **E:** Laminin (green) expression in the basement membrane surrounds ocular tissues in control eyes. Nuclei are counterstained with DAPI (blue). **F:** Persistent laminin expression in the ventral optic cup of *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* eyes (**arrow**). **G:** Colabeling of control ventral optic cup with VSX2 (red) and MITF (green). **H:** *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* ventral optic cup shows normal expression of VSX2 in retina and MITF in RPE. **I:** PAX2 expression (red) in ventral optic cup of control embryos. **J:** *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* ventral optic cup exhibits normal PAX2 expression. **K**–**N:** Optic cups at E10.5. **K:** Lateral view of Vax2 mRNA expression in ventral optic cup of control embryos. **L:** Vax2 mRNA is expressed in the *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* ventral optic cup (**arrow**). **M:** Tbx5 mRNA is expressed in the dorsal optic cup of control eyes. **N:** In *Porcn^{lox}/Y;Wnt1-Cre;Rx3-Cre* eyes, Tbx5 mRNA expression is slightly reduced in the dorsal optic cup (**arrow**). Scale bar = 100 μm.

cell death and proliferation are important processes during optic cup morphogenesis[84,87,88]; however, we observed no significant changes in the number of caspase-3 and p-histone H3–labeled cells in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes during the closure process (Supplemental Figure S3). Thus, RPE differentiation, dorsoventral patterning, apoptosis, and proliferation in the optic cup in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos show minimal changes, if any, and are unlikely to cause a defect in closure of the optic fissure. It is possible, however, that subsequent defects (eg, transdifferentiation of the ventral RPE) could contribute to formation of a wider gap in the optic fissure at E15.5 (Figure 3).

Next, we investigated whether changes in Wnt pathway activity occur in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes. The number of LEF1-positive cells was reduced in periocular mesenchyme underlying the ventral optic cup of *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos (Figure 6, A and B). We also examined expression of Axin-2 in the *Axin2*^{lacZ} knock-in reporter line[57] by detection of β-gal. In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos, β-gal expression was reduced in the mesenchyme underlying the optic fissure ($n = 5$) (Figure 6, C and D). Interestingly, β-gal expression was decreased in the RPE as well, consistent with transdifferentiation into retina at E15.5. Furthermore, Wnt–β-catenin activity is required for maintenance of PITX2, a key transcription factor that is robustly expressed in the periocular mesenchyme (neural crest and mesoderm) and required for anterior segment development.[65,89,90] Mutations in *Pitx2* cause Axenfeld–Rieger syndrome and can result in coloboma. However, PITX2 appears to be normally expressed in the periocular mesenchyme underneath the optic fissure of *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos (Figure 6, E and F). Furthermore, we observed no obvious changes in apical distribution of F-actin (Figure 6, G and H) or in expression of p-JUN (Figure 6, I and J). This suggests that apicobasal polarity and some aspects of noncanonical Wnt signaling appear to be normal in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos. However, the effects on LEF1 expression and *Axin2* reporter activation indicate that Wnt–β-catenin signaling in the ventral optic cup and underlying periocular mesenchyme is compromised.



Abnormalities in Anterior Segment Development in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* Eyes

Further analysis of *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos showed that eyelid morphogenesis and some aspects of anterior segment development can be disturbed (Figure 7). Because of perinatal lethality, it is not possible to examine formation of the chamber angle and trabecular meshwork, which develop postnatally (reviewed by Cvekl and Tamm[91]). Normally, in the optic cup periphery at E17.5, the ciliary body starts to fold and some extension of the iris epithelium is detectable (Figure 7C). In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes, pigmentation and early formation of ciliary body and iris are disturbed (Figure 7D), consistent

Figure 6 *Porcn* deficiency affects Wnt–β-catenin activity in the periocular mesenchyme underlying the optic fissure (**asterisks**), shown in sagittal views at E12.5. **A:** In control embryos, LEF1 is expressed in the periocular mesenchyme underlying the ventral optic cup, particularly in two to three cell layers underneath the optic fissure (**arrow**). **B:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos, LEF1 is expressed in fewer cells underneath the optic fissure (**arrow**). **C:** β-Galactosidase expression in *Axin2*^{lacZ} knock-in reporter embryos shows Axin-2 activation in the ventral retinal pigment epithelium (RPE) (**arrowhead**) and underlying mesenchyme (**arrow**). **D:** Activation of the *Axin2* reporter is reduced (**main image**) or absent (**inset**) in the ventral RPE (**arrowhead**) and mesenchyme (**arrow**) in mutant embryos. **E** and **F:** PITX2 is expressed in the periocular mesenchyme underlying the optic fissure in WT embryos (**E**) and appears normal in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos (**F**, **arrow**). **G:** Apical F-actin expression in the ventral optic cup of control embryos. **H:** F-actin expression shows a normal, continuous apical distribution in the *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* ventral retina and RPE (**arrow**). **I** and **J:** Expression of phosphorylated c-JUN (p-JUN) in the ventral optic cup did not differ obviously between control (**I**) and *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* (**J**) embryos. Scale bar = 100 μm.

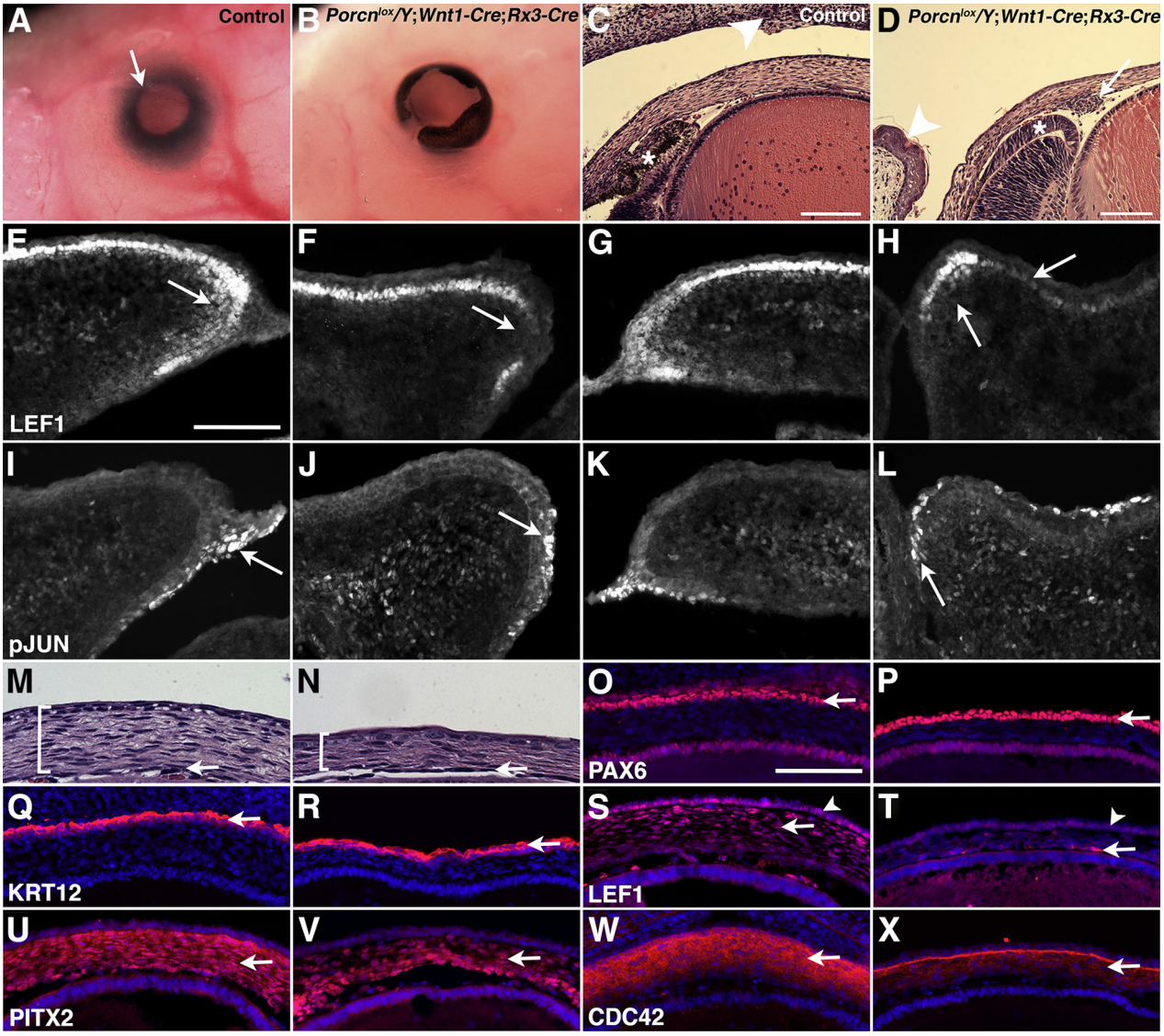


Figure 7 Eyelid closure defect and abnormal corneal development in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* mutants. **A:** Control embryo at E17.5 with closed eyelid (**arrow**). **B:** *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* mutant at E17.5 with open eyelid, coloboma, and pigment abnormalities in the optic cup periphery. **C:** Frontal section of the chamber angle of control eye at E17.5 with closed eyelid (**arrowhead**) and developing ciliary body and iris (**asterisk**). Hematoxylin and eosin stain. **D:** *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* anterior segment at E17.5 shows growth-arrested eyelid folds (**arrowhead**), abnormal development of the peripheral retinal pigment epithelium (RPE) in the absence of ciliary body and iris (**asterisk**), and ectopic cells in the chamber angle (**arrow**). **E:** LEF1 expression in palpebral epidermis, mesenchyme (**arrow**), and conjunctival epithelium in upper eyelid of control eye at E15.5. **F:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos, LEF1 expression in the palpebral epidermis and conjunctival epithelium of the upper eyelid is maintained, but is absent in the mesenchyme (**arrow**). **G:** Expression of LEF1 in the lower eyelid in control embryo. **H:** In the lower eyelid of *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos, LEF1 expression in the palpebral epidermis is decreased (**right arrow**), along with decreased expression in the mesenchyme (**left arrow**). **I** and **K:** Detection of p-JUN in the periderm, periderm extension (**arrow**), and conjunctival epithelium of upper (**I**) and lower (**K**) eyelids in control embryo at E15.5. **J** and **L:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyelids at E15.5, p-JUN appears to be normal (**arrows**). **M** and **N:** Histological cross section of the cornea at E17.5 in control (**M**) and *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes (**N**). Corneal thickness is indicated by a **bracket**; the corneal endothelium is marked by an **arrow**. **O**–**X:** Embryos at E15.5. **O:** PAX6 (red) expression in the corneal epithelium of control eyes (**arrow**). Nuclei are counterstained with DAPI (blue). **P:** PAX6 is present in the corneal epithelium in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes (**arrow**). **Q** and **R:** Expression of cytokeratin-12 in the corneal epithelium of control (**Q**) and *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* (**R**) embryos shows no difference (**arrows**). **S:** In control eyes, LEF1 is expressed in the corneal mesenchyme (**arrow**) and epithelium (**arrowhead**). **T:** LEF1 is decreased in the *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* corneal mesenchyme, except for some cells between lens and cornea (**arrow**). Reduced LEF1 expression in the corneal epithelium is variable (**arrowhead**). **U:** Robust PITX2 expression in control corneal mesenchyme (**arrow**). **V:** In *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* eyes, PITX2 is present in the hypocellular corneal mesenchyme (**arrow**). **W:** The Rho GTPase CDC42 is robustly expressed in the corneal mesenchyme in control eyes (**arrow**). **X:** CDC42 expression is severely reduced in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* corneal mesenchyme (**arrow**). Scale bar = 100 μm.

with the patterning abnormalities of the optic cup margins observed at earlier ages (Figures 2 and 3).

The eyelid closure defect in *Porcn*^{lox}*/Y;Wnt1-Cre;Rx3-Cre* embryos is completely penetrant (100%) (Table 1), and is not observed in conditional mutants with a single *Cre* allele, indicating that *Porcn* is required in multiple ocular surface tissues. Normally, between E11.5 and E15.5, the dorsal and ventral periocular ectoderm invaginate, and the resulting

eyelid folds grow toward each other across the surface of the eye. A projection of the outer, peridermal layer extends from the eyelid margins across the cornea until the periderm extensions meet and fuse. In *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* mutants, the periocular ectoderm has invaginated at E15.5 (Figure 3); however, growth of the eyelid folds is arrested and periderm extensions are not detectable (Figure 7, D–L). LEF1 is normally present in conjunctival epithelial cells, palpebral epidermal cells, and in mesenchymal cells, consistent with previous studies showing *Axin2* or *Tcf/Lef* reporter expression in developing eyelids (Figure 7, E and G).[92–95] In *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* embryos, mesenchymal expression of LEF1 is reduced or absent in both in upper and lower eyelids (Figure 7, F and H). In addition, LEF1 expression is decreased in palpebral epidermal cells of the lower eyelid (Figure 7H). We also examined p-JUN expression in the periderm and its extension, because it is required for eyelid closure.[96] p-JUN is detectable in the periderm of both upper and lower eyelids in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes (Figure 7, I–L). Overall, these results indicate that LEF1 expression in the eyelid mesenchyme is significantly affected by disruption of *Porcn*.

During normal morphogenesis of the cornea (approximately E12 in mouse), the periocular mesenchyme responds to cues from the lens and migrates into the space between lens vesicle and surface ectoderm, which matures into the corneal epithelium (reviewed by Cvekl and Tamm[91]). Migrating mesenchymal cells either undergo mesenchymal-to-epithelial transition and differentiate into corneal endothelium or differentiate into keratocytes, which produce the extracellular matrix of the corneal stroma. In *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes, the cornea separates from the lens (Figure 7, D and N), suggesting that maturation of the corneal endothelium proceeds normally. However, similar to *Porcn*^{het} eyes at E13.5 (Figure 2), we observed a thinning of the cornea in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes, caused by a severe decrease in cell number (Figure 7, M and N). The corneal epithelium is correctly specified in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes, because PAX6 and cytokeratin-12 are robustly expressed at E15.5 (Figure 7, O–R). This is consistent with previous studies indicating that Wnt–β-catenin activity is not required during differentiation of the corneal epithelium.[97,98] In the corneal mesenchyme, expression of LEF1 is markedly down-regulated, indicating reduced Wnt–β-catenin activity (Figure 7, S and T). However, similar to expression at E12.5, the Wnt–β-catenin target PITX2 is not affected by *Porcn* depletion; in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes, PITX2 is expressed in the hypocellular corneal mesenchyme at E15.5 (Figure 7, U and V). This suggests that differentiation of the hypocellular corneal mesenchyme at this age proceeds normally, despite reduced LEF1 expression. Furthermore, Rho GTPases can act as effectors of noncanonical Wnt signaling to regulate migration of the developing neural crest (reviewed by Mayor and Theveneau[99]). Specifically, the GTPase CDC42 is robustly expressed in the corneal mesenchyme, starting at E11.5, and regulates neural crest migration and/or proliferation during corneal morphogenesis and wound healing.[100–103] We observed that CDC42 was strongly expressed throughout the corneal mesenchyme in control eyes at E15.5, but was decreased in the *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* cornea (Figure 7, W and X). Taken together, our observations suggest that *Porcn* deficiency results in corneal hypoplasia due to defects in proliferation, survival, and/or migration of the periocular mesenchyme.

Discussion

Our present findings show that *Porcn* depletion during optic cup morphogenesis leads to closure defects of the optic fissure and eyelids, a hypocellular cornea, and a range of RPE defects. Unless *Porcn* is completely inactivated in most of the ocular and extraocular tissues, these defects are highly variable and less penetrant. This is reminiscent of ocular abnormalities found in female FDH patients, who exhibit mosaic deletion of *PORCN* due to stochastic X-chromosome inactivation. Approximately 40% of FDH patients exhibit ophthalmological findings, including microphthalmia, anophthalmia, coloboma (of the eyelid, iris, choroid, retina, and optic nerve), aniridia, and hypopigmentation.[1,2,16] Here, we have shown that deletion of *Porcn* in multiple tissues mimics several aspects of FDH with high incidence, and our analysis provides insight into some of the mechanisms by which *Porcn* regulates eye development.

Porcn Deficiency Causes RPE Abnormalities Characteristic of Atypical Colobomata and Aniridia

Porcn deficiency leads to loss of pigmentation in the optic cup periphery, and we identified tissue gaps or iris hypoplasia as one possible cause. These gaps could be thought of as atypical iris colobomata, because they arise outside of the inferonasal quadrant harboring the optic fissure. Atypical colobomata can occur independently from a closure defect in the optic fissure and are also associated with anterior segment disorders such as aniridia and Axenfeld–Rieger syndrome (reviewed by Chang et al[84]). Circumferential pigment defects are accompanied by tissue hypoplasia in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes, reminiscent of aniridia. These abnormalities are consistent with a role of Wnt–β-catenin signaling in differentiation of RPE, iris epithelium, and ciliary body in the optic cup.[24,27,30] Deletion of β-catenin in the optic cup periphery attenuates growth and patterning of the ciliary margin, accompanied by a shortened optic cup.[24,30] Similarly, combined disruption of the secreted Wnt modulators sFRP-1 and sFRP-2 (which are required here for extracellular spreading and availability of Wnt proteins) leads to identical defects.[19,78]

A considerable number of *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes exhibit severe transdifferentiation involving major parts of the proximal RPE; to date, this has not been described in humans. Previous studies, including our own, have

demonstrated that a complex of β-catenin−TCF/LEF directly transactivates enhancers of the RPE key regulatory genes *Mitf* and *Otx2*; thus, deletion of β-catenin in the RPE leads directly to a loss of MITF and OTX2, which are strictly required for RPE development.[20,21,25,29] Our present findings provide further evidence that it is the signaling function of β-catenin that is required to promote RPE differentiation. Interestingly, our findings also suggest that Wnt proteins secreted from multiple tissues are required for Wnt−β-catenin activation in the RPE, because *Porcn* depletion in periocular mesenchyme or optic neuroepithelium or lens alone is not sufficient to cause transdifferentiation of RPE into retina.

We observed that, in rare cases, transdifferentiation involving large regions of the posterior RPE resulted in extreme RPE defects during optic cup formation (Figure 3H), possibly because of an earlier onset of *Porcn* inactivation. It is conceivable that this might later lead to severe microphthalmia; however, we could not explore this because of perinatal lethality. Furthermore, *Porcn* depletion before the optic vesicle stage could result in extreme microphthalmia or anophthalmia. Noncanonical Wnt signaling is required before optic vesicle formation in zebrafish and frog, either to suppress canonical Wnt pathways and/or to promote eye-specific gene expression[41,43,44] (reviewed by Fuhrmann[104]), and mutation of PCP effector genes such as *Fuz*, *Int*, or *Wdpcp* results in microphthalmia or anophthalmia in mouse.[45−47] Thus, the occurrence of FDH with severe microphthalmia or anophthalmia could be explained by an early role of noncanonical Wnt signaling during eye development.

Porcn Regulates Closure of the Optic Fissure

Similar to FDH in humans, in mouse the loss or reduction of PORCN results in a failure of the optic fissure to close. We found that the neuroepithelium in the ventral optic cup and underlying periocular mesenchyme differentiated properly up to E12.5, because several genes critical for closure of the optic fissure (eg, *Vax2*, *Pax2*, *Mitf*, and *Pitx2*) were normally expressed. Maintenance of PITX2 expression is transiently dependent on Wnt−β-catenin signaling,[89] and so the normal expression pattern of PITX2 in *Porcn^{lox}/Y*;*Wnt1-Cre*;*Rx3-Cre* mesenchyme was unexpected. This suggests that the temporal requirement for Wnt−β-catenin activity may have passed already. Furthermore, neural crest−specific inactivation of β-catenin causes abnormalities in the optic stalk and eye positioning; however, it is not clear whether the optic fissure closes properly.[89] Further studies are needed to determine whether the closure defects results from impaired Wnt−β-catenin activity in the mesenchyme.

Disruption of the Wnt receptor Frizzled-5 (encoded by *Fz5*) during early embryonic development occasionally causes coloboma, and the incidence of coloboma increases when one allele of the closely related *Fz8* is additionally removed.[77,81] These triallelic *Fz5/Fz8* mutants exhibit retinal neurogenesis defects that are attributed to abnormal formation of retinal apical junctions and defective HES1 expression, consistent with a role of *Fz5* and *Fz8* in neuronal polarity and possibly noncanonical Wnt signaling during neural development.[77,105] However, it has not been determined whether apical junction formation is already impaired during closure of the optic fissure in triallelic *Fz5/Fz8* mutants.[77] *Porcn^{lox}/Y*;*Wnt1-Cre*;*Rx3-Cre* embryos do not exhibit obvious defects in retinal neurogenesis and HES1 expression, and apicobasal polarity appears to be normal in the ventral optic cup. One possibility is that FZ5 and FZ8 are activated by ligands independently of Wnt proteins. On the other hand, there is increasing evidence that interference with noncanonical Wnt signaling can result in ectopic activation of Wnt−β-catenin signaling,[52,106−109] and disruption of the canonical Wnt antagonists Dkk-1 and Axin-2 results in coloboma[110] (A.A. and S.F., unpublished data). It is possible, therefore, that colobomata in triallelic *Fz5/Fz8* mutants result from ectopic activation of Wnt−β-catenin signaling.

Disruption of the Wnt coreceptor LRP6 causes severe coloboma and dorsoventral patterning defects; specifically, the dorsal domain of the optic cup is dramatically reduced, as shown by loss of Tbx5 and expansion of Vax2 expression.[82] By contrast, in *Porcn^{lox}/Y*;*Wnt1-Cre*;*Rx3-Cre* embryos, colobomata are less severe, characterized usually by a small gap ventrally, and dorsoventral patterning appears largely normal. Although disruption of *Porcn* is expected to interfere with all Wnt pathways, this difference could be explained by unexpected, PORCN-independent secretion of some Wnt proteins. Another possibility is that *Porcn* deletion in our *Porcn^{lox}/Y*;*Wnt1-Cre*;*Rx3-Cre* embryos is not complete. Interestingly, recent studies have identified novel, highly context-dependent functions of LRP6; although it is required to activate Wnt−β-catenin signaling, LRP6 modulates the noncanonical Wnt/PCP pathway during mouse heart morphogenesis and neural tube closure.[106,107] These and other studies show that the outcomes of LRP6 and Wnt signaling are highly context-dependent and can involve antagonistic or coregulative action of both Wnt−β-catenin and noncanonical Wnt pathways. Thus, it is possible that *Lrp6* mutants may exhibit gain of function of noncanonical Wnt signaling during closure of the optic fissure, and we hypothesize that this could exacerbate defects in optic cup morphogenesis.

Defects in Eyelid Closure and Corneal Morphogenesis on *Porcn* Disruption

FDH can manifest with coloboma of the eyelids, and our observations in *Porcn* mutants are consistent with a role of Wnt signaling during eyelid morphogenesis and closure.[13] LEF1 expression in the eyelid mesenchyme and eyelid epithelium is decreased, indicating compromised Wnt−β-catenin signaling. Defects can manifest as permanent open eyelids or delayed closure, and disruption of several Wnt−β-catenin and noncanonical pathway genes (eg, *Tcf3*, *Dkk2*, *Lrp6*, *Vangl2*, *Fz3*, *Fz6*, *Celsr1*, and *Ptk7*) causes eyelid closure abnormalities.[95,98]

Surprisingly, the actual role of Wnt signaling in eyelid closure is not well understood. A detailed analysis in mouse using *in vivo* imaging in combination with genetic and laser ablation revealed that epidermal cells intercalate and generate a force towing the surrounding epidermis over the eye.[94] These movements appear similar to convergent extension movements in gastrulation, which are mediated by noncanonical Wnt signaling. It is therefore possible that localized actions of both Wnt−β-catenin and noncanonical Wnt signaling participate in growth and fusion of eyelids.

We found that *Porcn* deficiency leads to severe hypoplasia of the corneal mesenchyme in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* eyes. PITX2 is expressed in the hypocellular cornea, suggesting that the remaining mesenchymal cells may differentiate normally. Other processes of periocular mesenchyme development that could be affected by *Porcn* deficiency at this stage are survival, proliferation, and migration. Such defects may explain the abnormally reduced primary vitreous body in *Porcn*-mutant embryos. The Rho GTPase CDC42 can act downstream of noncanonical Wnt signaling and has been implicated in migration and proliferation of cranial mesenchyme during development and wound healing of corneal endothelium.[100−103] We observed decreased expression of CDC42 in the hypocellular corneal mesenchyme in *Porcn*^{lox}*/Y*;*Wnt1-Cre*;*Rx3-Cre* embryos. Further studies are needed to determine whether the decrease in CDC42 expression is a result of abnormal noncanonical Wnt signaling. In addition, the decrease in LEF1 expression suggests that Wnt−β-catenin signaling may be affected, therefore, we cannot exclude that distinct Wnt pathways participate in regulating morphogenesis of the cornea.

In summary, we have presented a mouse model that shows high penetrance of ocular defects reminiscent of those found in FDH. Our results demonstrate that *Porcn* is required in multiple tissues to regulate distinct processes during embryonic eye development. We also identified a novel role for PORCN/Wnt signaling in corneal morphogenesis. Our results show that Wnt proteins from multiple tissues mediate critical interactions among periocular mesenchyme, surface ectoderm, and optic cup neuroectoderm, consistent with activity of potential Wnt pathway readouts (LEF1, AXIN-2, CDC42). Further studies are needed to determine the actual molecular and cellular mechanisms underlying the ocular defects caused by *Porcn* deficiency.

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Supplemental Data

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